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THE MECHANISMS OF THROMBIN FORMATION

S. Béguin, P.P. Devilée, H.C. Hemker

Department of Biochemistry, University of Limburg, Biomedical Center, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

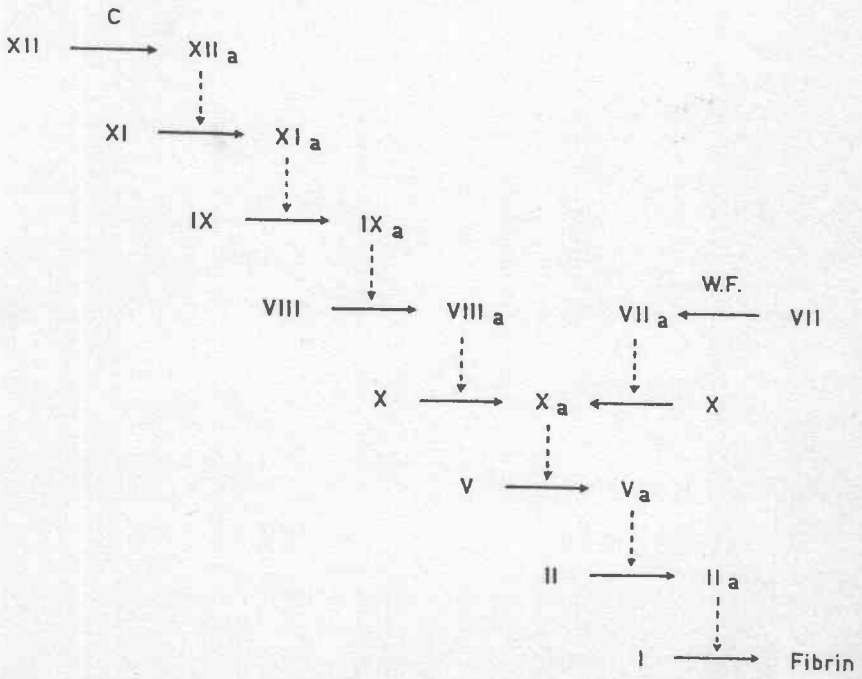

Fig. 1 The classical coagulation cascade (Macfarlane 1969)

S. Dawids (ed.), Polymers: Their Properties and Blood Compatibility, 81–104.

Thrombin is the pivotal enzyme of haemostasis and thrombosis. It catalyzes many important reactions in these processes among which coagulation in the strict sense of the word, i.e. the enzymatic conversion of fibrinogen in fibrin by thrombin. The formation of thrombin in the blood results from a complicated series of chemical and physical interactions; its subsequent inactivation also. In vivo these opposite mechanisms (activation-inactivation) are tuned so precisely that the blood remains fluid in the vessels but any leak is promptly mended. When this equilibrium is disrupted either bleeding or thrombosis will ensue.

Because of the enormous progress in modern coagulation biochemistry, resulting in the isolation and purification of the proteins of coagulation and detailed enzymological studies on their interactions, it is readily possible at this moment to give a plausible scheme of the biochemical reactions which contribute to the formation and to the disappearance of thrombin. Especially in the last ten years, kinetic studies of isolated steps of the coagulation mechanism revealed many details of the biochemical mechanisms involved. There is a consensus on the existence of two pathways that explain the mechanism of the coagulation of blood: the intrinsic pathway operative when coagulation is started by contact of blood with glass or other foreign surfaces, and the extrinsic pathway triggered by the addition of tissue thromboplastin. In both cases, calcium is essential. However, these standard schemes of blood coagulation proposed for in vitro situations will not necessarily apply in vivo. Thus some questions from the patho-physiology of blood coagulation remain even today without answer, e.g.: How to explain the importance of the intrinsic pathway in vivo in the obvious absence of foreign surfaces? or: Why do haemophiliacs bleed whereas the proband of Factor XII deficiency, Mr. Hageman had no bleeding problems and died of thrombosis? or: Why is it that heparin hardly influences the thromboplastin time and in the same concentration has a marked influence both on the thrombin

time and the activated partial thromboplastin time? again: Why do some kinds of thrombopathies show clotting disorders whereas most do not? and: How to explain the origin of early traces of thrombin necessary to activate Factors V and VIII? The answer to these questions does not readily follow from the classical coagulation schemes. Other interactions between the clotting factors must exist. In the course of the years many accessory reactions, not featuring in the classical schemes ("cross reactions") have been demonstrated to be possible in vitro but it remains difficult to see which of them are of importance in vivo and which are not.

Already in 1976, Hurlet-Birk Jensen, Josso and Béguin produced evidence for thrombin formation in the very early stage of haemostasis before clotting occurred. This was done by means of the measurement of the activation of factors V and VIII in the blood flowing from a wound in normal and congenitally deficient subjects. This type of experiments requires the presence of patients in the laboratory, because the activation of the factors is not stable and difficult to quantify if the determinations are not carried out immediately. This is one example of the importance of close collaboration between the clinics and the research laboratory for the study of haemostasis and thrombosis. The older literature abounds with more examples but even today, patients like those exibiting abnormal prothrombin (prothrombin Metz, prothrombin Barcelona), abnormal antithrombin III (AT III Alger) or the Scott thrombopathy show that the experiments of nature, that can be found by attentive physicians among the patients under their care, are never to be neglected. In general, the (patho)physiology of haemostasis and thrombosis has a wider scope than the study of interactions of isolated coagulation factors. This has to be kept in mind while reading a review on coagulation biochemistry.

The basis of the physiology of the coagulation were established in the second part of XIXth century. After the discovery of thrombin (Buchanan 1836-1845, Schmidt

1861-1892) the definition of fibrinogen (Virchow 1856; Denis 1859) and its isolation by Hammarsten (1876-1880), the demonstration of the important role of calcium in coagulation (Arthus and Pages 1890) and finally the discovery of "prothrombin" by Pekelharing in 1895, Morawitz in 1905 proposed the first coherent model of coagulation, which will then be maintained during some forty years. In 1940 Seegers purified "prothrombin" (i.e. what is now known to be a mixture of the factors II, VII, IX and X). This opened the era of modern biochemical coagulation research. Yet, still in 1947, only two proteins of coagulation were sufficiently defined: fibrinogen and prothrombin. In that year Owren published his study on Factor V (proaccelerin). Concomitantly Owren created a methodology which allowed the discovering of ten clotting factors. In 1964, the hypothesis of a "cascade" of enzymatic reactions was advanced by Mac Farlane. This was the trigger for many enzyme mechanistic studies that led to the establishment of a reaction sequence that we will discuss now.

A. THE CLASSICAL COAGULATION MECHANISM.

1. Activation by limited proteolysis.

The mechanism of activation by limited proteolysis is central in the blood coagulation reaction sequence. Studies on digestive proteolytic enzymes ((chymo)-trypsin, pepsin etc) and their zymogens have made proenzyme $\longrightarrow$ enzyme conversions of proteolytic enzymes one of the main subjects of classical enzymology (1,2). The main chain of tissue thromboplastin induced proteolytic activations in blood is: *

$$VII \longrightarrow X \longrightarrow II$$

For intrinsic coagulation the main chain of activations is (3):

$$PK \rightleftharpoons XII \longrightarrow XI \longrightarrow IX \longrightarrow X \longrightarrow II$$

All the non-activated clotting factors participating in these chains are proenzymes of serine proteases, the activated enzymes consequently are serine proteases.

2. Heterogenous biocatalysis.

The proteolytic activations shown above can be obtained when the molecules encounter in free aqueous solution. They are accelerated up to 100.000 fold however by the presence of a phospholipid interface and specific protein cofactors. To illustrate this mechanism we will take the activation of prothrombin as an example. Factor X_a is capable to generate thrombin from prothrombin in free solution, but only in a very ineffective mechanism (4,5,6). Hanahan & Papahadjopoulos (7) were the first to observe that an active prothrombinase exists only in preparations that contain the three components FX_a, FV and phospholipid. (The question of factor V activation will be discussed later). Hemker et al (8) showed that the generation of prothrombinase activity can be described as the reversible formation of a complex of FX_a, FV and phospholipid. In a series of very elegant experiments Rosing et al (9) later showed that phospholipids diminish the K_m for prothrombin

* Footnote:
Arrows indicate activation steps, double arrows mean mutual activation. Brackets indicate multienzyme complexes.
PK = Prekallikrein, TF = Tissue Factor, PL = Phospholipids.
Roman numerals indicate the factors. II = prothrombin.

conversion (a typical change would be from 3000 nM to 30 nM) whereas Factor V_a increases the turnover number (k_{cat}) about 1000 fold.

Further investigations (9-16) showed that the change in K_m is caused by the fact that the lipid bound enzyme has higher affinity for the substrate then the free enzyme has. The change in k_{cat} is probably brought about by an alignment of the active-site of Factor X_a to the vulnerable sites of Factor II caused by their mutual interaction with Factor V_a.

In 1967 Hemker and Kahn (17) found that the Factor X activating enzyme is a complex of the Factors $VIII_a$ and IX_a and phospholipid, completely comparable to the prothrombinase complex. Later, van Dieijen et al (18) showed that in this complex the kinetic effects of phospholipids (on K_m) and Factor $VIII_a$ (on k_{cat}) were similar to those of phospholipid and Factor V_a in the prothrombinase complex. Apart from their kinetic effect on k_{cat}, the factors V_a and $VIII_a$ also serve to better bind their respective enzymes (FX_a and FIX_a) to phospholipid (19,20).

The available data, primarily those coming from the laboratory of Nemerson (21) indicate clearly that Factor VII and tissue thromboplastin form a complex that is again comparable to prothrombinase. In this case the protein cofactor and the phospholipid are intimately bound but the mechanistic role of the protein cofactor, like Factor V_a, seems to be to enhance the efficiency of the enzyme whereas the lipid serves to booster the affinity for the substrate. The most obvious difference with the other complexes resides in the fact that tissue thromboplastin does not arise from an on the spot combination of the protein cofactor and the lipid but is a tight lipoprotein complex, shed as such by wounded cells. We will not enlarge upon the surface reactions and cofactors of contact activation here for reasons that will be discussed later.

We can summarize the reactions of the classical coagulation pathways as follows:

Extrinsic pathway:

$$(VII, T.F., PL) \rightleftharpoons (X, V_a, PL) \longrightarrow II$$

Intrinsic pathway:

$$PK \rightleftharpoons XII \longrightarrow XI \longrightarrow (IX, VIII_a, PL) \longrightarrow (X, V_a, PL) \longrightarrow II$$

3. Limitation of thrombin formation

An overview of the coagulation reactions is not complete if no account is given of the way in which thrombin formation is limited. Apart from the trivial possibility of substrate exhaustion, more often then not silently assumed to be the clotting delimiter in the older literature, there are two main mechanisms to be considered: scavening of coagulation proteases by antithrombin III, α_2Macroglobulin and other antiproteases ($\alpha_1$antitrypsin) (see ref. 22 for a review) and breakdown of the protein cofactors (FV_a and $FVIII_a$) by activated protein C (together with protein S) (23,24). Protein C and protein S are vitamin K dependent proteins circulating in the plasma, (25,26). Protein C is activated by thrombin adsorbed onto thrombomodulin (27). Because thrombomodulin occurs at the surface of intact endothelium this mechanism may help to limit thrombin formation to wounded areas.

Among the anti-proteases, AT III is extremely important because of the fact that its activity can be enhanced by heparins (28-30) which makes it the lever on which this important family of antithrombotic drugs acts.

B. FEEDBACK REACTIONS

1. Activation of factors V and VIII by thrombin.

It has been shown that both Factor VIII and Factor V have to be activated before they can play their role as a protein cofactor, and that thrombin is the enzyme that brings about these activations (31-34). Thrombin-activated Factor V ($F.V_a$) in a purified state, when kept under the right conditions is relatively stable. The active state of factor VIII always seems to be a transient phenomenon, ending in inactivation. The activation of Factor V has been described in terms of protein chemistry. The single chain molecule of Factor V is cleaved in three places by thrombin and two of the four resulting fragments recombine under the influence of Ca^{++} to form Factor V_a. The mode of interaction between Factor VIII and thrombin probably is a similar process (35). It has been described that in a human system Factor X_a can activate Factor VIII (36).

At this moment it is established beyond any reasonable doubt that activation of the Factors V and VIII is obligatory for their taking part in the coagulation mechanism. This does not mean however that we know wether these reactions are physiologically important. To be more exact: it is essential to know wether these activations play a rate limiting role under physiological conditions and what the physiological activator is. From the work of Hurlet et al (37) it may be concluded that the activation of V and VIII does occur *in vivo* in the time course of normal haemostasis. An aberrant prothrombin that possibly yields a thrombin incapable of activating Factor V, prothrombin Metz (38,39) causes a mild bleeding disorder. Recent results obtained by the authors and to be published elsewhere indicate the feedbacks activation of factor VIII is rate limiting in the intrinsic pathways, wheras that of factor V is not.

2. Activation of platelets by thrombin.

Thrombin is the most potent physiological platelet activator known (40), a concentration of 0.1 to 1.0 nM will suffice to trigger a half maximal release reaction. All other activators of human platelets need concentrations that are one or more orders of magnitude higher if they are to cause the same response.

Among the proteins released by platelets are Factor V and heparin neutralizing proteins (platelet factor 4). The amount of Factor V sequestered in the platelets is roughly 20% of the amount present in the plasma (41). Thrombin, that causes the release reaction will also activate the released Factor V. It has been shown that this activation rather then the release reaction itself is the rate limiting factor for the generation of Factor V activity from triggered platelets (42). The concentration of Factor V in platelet poor plasma is about 25 nM whereas that of its partner, Factor X is around 200 nM. This may lead one to think that the contribution of platelet Factor V may be important in vivo. The aggregation of platelets at sites where the hemostatic mechanism is active will cause a further increase in the ratio of platelet-Factor V to plasma-factor V. Still patients with a storage pool deficiency that are unable to release Factor V from their platelets do not have an important hemorrhagic diathesis (43). It seems that only patients lacking Factor V in both platelets and plasma do show a hemorrhagic syndrome (44). This may be explained by the generally recognized fact that the normal level of any clotting factor represents a large functional excess. As a rule the level of any clotting factor must drop significantly below 10% before a decrease of the clotting function becomes apparent.

A second procoagulant function of platelets induced by thrombin together with collagen is the platelet "flip-flop" reaction discovered by Bevers et al. (45).

This reaction consists of a transbilayer movement of the procoagulant, negatively charged phospholipids (primarily phosphatidyl serine) that as a rule are to be found almost exclusively at the inner face of the cell membrane. In the presence of collagen and thrombin, platelets produce these procoagulant phospholipids at the outside of the cell without the cell being disrupted. The precise molecular mechanism of this reaction is not yet clear. Anyhow, platelets thus activated, offer large amounts of binding sites for the Factors IX_a, $VIII_a$, X_a and V_a at their outer surface so that prothrombinase and the Factor X activating enzyme can readily form there. One patient (Mrs. Scott, USA) has been described in which this mechanism is defective, she suffers from a mild haemorrhagic diathesis consequently known as Scotts syndrome.

It has been reported that collagen-activated platelets can start coagulation via a Factor XI dependent mechanism and that ADP activation of platelets triggers coagulation via Factor XII (47). These findings remain to be confirmed. The recent observation that platelets release a potent inhibitor of Factor XI_a, so that Factor IX activation by Factor XI_a hardly proceeds in the presence of activated platelets (48) makes one doubt the importance of contact activation for <u>in vivo</u> thrombin generation.

3. <u>The Josso loop</u>.

In the classical view, contact factors and antihemophilic factors form the intrinsic pathway of thrombin formation and the importance of the role of the contact factors is derived from the recognized importance of the antihemophilic factors. The activating action of Factor VII on Factor IX invalidates this argument. The first indications that the action of the antihemophilic factors (FVIII and FIX) is not confined to the coagulation pathway started by the contact factors were obtained by

Biggs and Nossel (49). Josso (50) was the first to postulate that Factor VII can activate Factor IX so that the antihemophilic factors play a role in thromboplastin triggered coagulation. This means that Factor X can be activated either directly by Factor VII and tissue thromboplastin or indirectly by Factor IX_a (together with Factor $VIII_a$) that, in its turn has been activated by Factor VII (see fig. 1). It is easy to see that the function of this pathway will anyhow be dependent upon the amount of thromboplastin available. The contribution of the direct, one-step action of Factor VII_a on Factor X_a formation will be constant in time and roughly proportional to the concentration of thromboplastin. The contribution via the pathway VII $\longrightarrow$ IX $\longrightarrow$ X will be small in the beginning of the reaction but will increase proportionally with time as the Factor X activating enzyme (i.e. Factor IX_a) builds up. Therefore the reinforcement loop constituted by the antihemophilic factors - which we proposed to call the Josso loop after its discoverer (50) - will gain in importance when clotting is started by smaller amounts of thromboplastin. The early observations on the interconnections between the extrinsic and the intrinsic pathway did not get the attention they deserved until Østerud and Rappaport drew attention to the fact that the Factor VII- thromboplastin complex is capable of activating Factor IX in a partially purified system (51). Later Zur and Nemerson (52) Jesty and Silverberg (53) and Marlar and Griffin (54) established this pathway without any reasonable doubt.

The physiological importance of the Josso loop is difficult to ascertain because of the thromboplastin dependent and hence time dependent effect discussed above. It is tempting to use the Josso-loop mechanism as a tentative explanation for the clinical observation that hemophiliacs tend to bleed in thromboplastin poor organs such as joints, but this can hardly be accepted as a proof of its importance. Jesty and Silverberg (53)

calculate that the activation of Factor X by Factor VII_a is 6 to 7 times faster than the activation of Factor IX. Zur and Nemerson (52) find a ratio of 10 of the theoretical maximal velocities but argue that the actual ratio will be completely dependent upon the thromboplastin concentration. Van de Besselaar et al. (55) conclude from observations in deficient human plasmas that the Josso loop may be of no importance in human plasma. Clearly the issue is not settled as yet. Recent unpublisehd work from the present authors demonstrates that at low concentations of tissue thromboplastin the Josso loop contributes significantly to thrombin generation in whole plasma.
Kalousek et al. (56) have reported that Factor X is able to activate Factor IX. This would constitute a mutual activation interaction that could enhance Factor X activation even without activation of Factor IX by Factor VII. Their experiments have been carried out in purified systems that did not contain protein cofactors (F.V anf F. VIII). Any indication as to the physiological significance of this interaction is lacking at this moment.

4. Activation of Factor VII.

The current view on the starting mechanism of coagulation is based on the observation that the proenzyme Factor VII has a non-neglectible enzymatic activity (57,58). Once it adsorbes onto tissue thromboplastin, the activity of Factor VII is enhanced so as to become sufficiently important to start the clotting process. It has been observed however that there exists a more active form of Factor VII, the two chain Factor VII_a. This form can be generated from the one chain form in a number of different ways. Altman and Hemker (59) showed, as early as 1967, that the contact activation mechanism can enhance Factor VII activity in vitro. The cold activation of Factor VII, involving kallikrein and different other

proteins has been well established. It has also been described that Factor VII can be activated by Factor IX_a and by Factor X_a (60,61). A very interesting suggestion is made by Silverberg and Jesty (62), when they claim that a complex of Factor VII, tissue thromboplastin and Factor X_a in the proteolytically active species.

If anywhere, then it is at the level of the activation of Factor VII that every conceivable reciprocal interaction of clotting factors has been described whereas any indication of their physiological importance is lacking. It is evident that all biochemical observations do not necessarily represent reactions that play a role in (patho) physiology. This being said, it must also be mentioned that often conclusions are drawn too quickly from clinical observations. Tradition has it that the scarce observations of a Factor VII deficiency or of any other rare clotting factor deficiency provoke speculations as to the physiological importance of a deficiency, of that specific factor. Now some observe a low Factor VII level ($< 5\%$) without clinical symptoms whereas others find these patients severely handicapped. The same holds for Factor XI deficiencies and others. In trying to interprete these data one should be aware of the following:

a) Any really important bleeding syndrome will lead to death either before or shortly after birth. Only the relatively mild syndromes survive. We remind of the analogy in thrombophilia: AT III and protein C deficiencies are very rarely seen in the homozygous states, probably because complete deficiencies are lethal to the foetus.

b) Any deficiency that does not lead to a clinically important syndrome will more often then not go unnoticed. It must be kept in mind that the physiological levels of clotting factors as a rule represent a large excess of that factor so that a decrease to as low as $\sim$ 10% of the normal level will not cause any overt disease. The number

of deficiencies that are recognized not to cause problems will therefore depend on chance findings and hence be under-estimated. This is illustrated by the fact that these disorders tend to cluster around laboratories that specialize in research on the blood coagulation and that are backed up by competent clinicians. We thus see that neither the really important deficiencies nor those without any clinical consequences will be recognized in routine medical practice. Therefore it is very hard - if not impossible - to draw conclusions on the mechanism of the blood coagulation process from the correlation between observed clinical symptoms and the accompanying clotting factor deficiencies.

5. INHIBITORY FEEDBACK REACTIONS

One may think of several crosslinks between the reactions that inhibit the clotting process and those that enhance thrombin formation.

The thrombin feedback reactions play an important role here. As we have discussed before, thrombin will enhance its own formation by activating the factors V and VIII as well as platelets. Any inhibition of thrombin formation and any reaction that inactivates thrombin therefore will interfere with this positive feedback. The interesting question remains in howfar antithrombin III inhibits thrombin formation because it modulates the extend of activation of Factor V or Factor VIII etc. We recently showed that this mechanism is operative in the intrinsic system (unpublished). Because heparin acts via antithrombin III, this means that heparin influences factor VIII dependent coagulation pathways indirectly by inhibiting the feedback activation of factor VIII. Pentosan polysulphate, a heparinlike drug, acts by direct inhibition of factor VIII activation (Wagenvoord et al., unpublished). Also diminution of the available amount of thrombin will cause a decreased rate of activation of

protein C and therefore prolong the mean lifetime of the prothrombin and Factor X activating complexes. Again no quantitative data are available that allow an estimate of the importance of this conceivable pathway.

In the second place one has to reckon with competition for clotting proteases between procoagulant complexes and protease scavengers. Here more data are available. It has been observed for instance (63,64) that antithrombin III will attack Factor X_a less readily in the presence of phospholipids then in free solution and even less if both phospholipids and Factor $V_{(a)}$ are present. In vivo this protection seems even to be complete (unpublished results). What holds for Factor X_a and antithrombin III, in principle holds for all surface bound proteases and all antiproteases. Lipid binding of Factor IX_a protects this enzyme too from antithrombin III-heparin action.

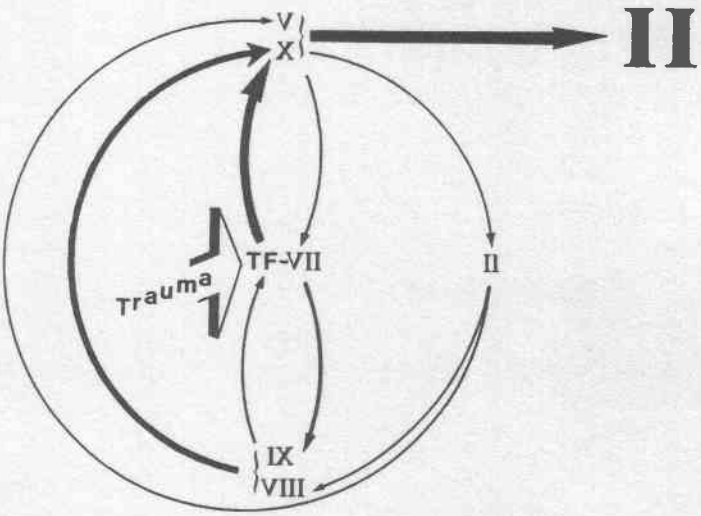

Fig. 1 A coagulation scheme for 1988
The thickness of the lines indicates roughly the rate of the reactions.

TABLE I

CONCENTRATIONS OF SOME PLASMA PROTEINS

Abreviations		Molecular weight kilo dalton	Approximate conc. in plasma (nMola
F.XII	Factor XII	80	450
F.XI	Factor XI	170(dimer)	30
F.IX	Factor IX	70	70
F.VIII	Factor VIII	200	0.5-1
F.VII	Factor VII	45.5	10
F.X	Factor X	55	180
F.V	Factor V	300	25
F.II	Factor II	66	1500
F.XIII	Factor XIII	350	
F.I	Fibrinogen	360	10000
P.K.	Prekallikrein	100	1100
HMWK	High molecular weight kininogen	200	350
PC	Protein C	60	66
PS	Protein S	70-85	130
P.gen.	Plasminogen	92	1400
AT III	Antithrombin III	65	2000
HC II	Heparin Co-factor II	65-70	1200-450
$_2$ AP	α -antiplasmin	65	900
$_2$ M	α -macroglobulin	750	3500
$_1$ AT	α -antitrypsin	52	25000
	inter-α-trypsin inh.	160	3000
	C^1-inhibitor	150	2200
	S-protein	74	6700

Table II

Thrombin concentrations

1 N/H unit = 0.324 ± 0.073 ug

1 N/H unit = 8.85 ± 2.01 pMole

1 U/ml ≙ 10 pMole/ml ≙ 10 nMolar

1 IOWA unit = 0.83 N/H units

1 WHO unit = 0.56 N/H units

Table III

Heparin concentrations

1 Unit ≙ 5.5 ug

1 ug ≙ 0.18 unit

M.W. ≙ 15.000

Unit/ml ≙ 400 nMole/L

The values differ from batch to batch

The molecular weight is a rough estimate of the mean molecular weight.

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